발생과 생식 제 3 권 제 1 호, 45~51 (1999)
Dev. Reprod. Vol. 3, No. 1, 45~51 (1999)

Efficient Establishment of Presumptive Embryonic Stem Cells from Bovine Blastocysts by Exposure to Calcium Ionophore

Sun-Uk Kim, Zae-Yoong Ryoo[1], Chul-Sang Lee, Yong-Man Han, Jung-Sun Park, Dae-Yeul Yu and Kyung-Kwang Lee

Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Taejon 305-600, Korea

[1]Research Institute of Experimental Animals, Catholic Research Institutes of Medical Science, Catholic University of Korea, Seoul 137-040

Calcium Ionophore를 이용한 소 배반포로 부터의 배아주 유사세포의 효과적인 분리

김선욱 · 류재웅[1] · 이철상 · 한용만 · 박정선 · 유대열 · 이경광

생명공학연구소, [1]가톨릭 의과대학 의과학연구원 실험동물학연구실

ABSTRACT : It is crucial to remove trophectoderm (TE) cells of blastocysts for an efficient isolation of pluripotent embryonic stem (ES)-like cells from bovine blastocysts. We evaluated the effectiveness of chemosurgery using calcium ionophore A23187 (CIPA) by investigating the viability and pluripotency of ES-like cell lines isolated from *in vitro*-produced bovine blastocysts after CIPA treatment. The blastocysts treated with 50 μM CIPA for 25 min colonized most efficiently (51% of blastocysts) and developed to ES-like cell lines through 10 passages (4.8% of blastocysts) among CIPA-treated groups with different concentration and duration. In comparison with CIPA-untreated blastocysts, the colonization rate and overall viability of the CIPA-treated blastocysts were five times higher, suggesting that CIPA treatment condition defined in this study was highly efficient for establishing ES-like cell lines without apparent toxicity of CIPA. We evaluated *in vitro* pluripotency of the established three ES-like cell lines by examining alkaline phosphatase (AP) activity, capability of embryoid body formation, and chromosomal euploidity of the cells. Our cells showed a heterogeneous AP activity similarly to other reports. The cells were able to form simple embryoid bodies during suspension culture and majority of them showed a normal chromosome number of 60, the euploid chromosomal complement of bovine. Therefore, our data suggest that CIPA treatment can be safely used for an efficient isolation of ES-like cell lines from bovine blastocysts.

Key words : Bovine blastocyst, Calcium ionophore A23187, ES-like cell lines.

요 약 : 소 배반포로부터 배아주 (embryonic stem, ES) 유사세포를 분리하기 위해서는 영양외배엽 (trophectoderm, TE) 세포를 제거하는 것이 효과적이다. 따라서 본 실험은 효과적으로 TE를 제거하기 위한 calcium ionophore A23187 (CIPA) 처리조건을 확립하고, 분리해낸 ES 유사세포의 *in vitro* 다능성 (pluripotency)을 검증하고자 수행하였다. CIPA 농도 및 처리시간을 달리 하였을 때 50 μM에서 25분간 처리한 군이 colony 형성율 (51%) 및 10 passage 까지의 배양성적 (4.8%)에서 가장 좋은 결과를 나타내었다. 또한 CIPA를 처리하지 않은 군과의 비교에서도 약 5배의 높은 결과를 보임으로서 본 실험에서 확립된 CIPA 처리조건은 가시적인 toxicity 없이 ES 유사세포의 확립에 이용될 수 있음을 시사하였다. 확립된 ES 유사세포는 heterogeneous한 alkaline phosphatase (AP) 활성을 보여 소 ES 유사세포에 대한 타 보고들과 유사한 결과를 보였다. *In vitro* 부양배양 (suspension culture)에서는 embryoid body로 분화가 가능하였으며, 약 70% 정도의 euploidism을 보였다. 따라서 본 실험에서 확립된 CIPA의 처리조건이 소 배반포로부터 ES 유사세포를 확립하는데 효과적으로 이용될 수 있음을 확인할 수 있었다.

INTRODUCTION

Murine embryonic stem (ES) cells are pluripotent and can contribute to somatic and germinal development when they are introduced into the preimplantation embryos (Evans & Kaufman, 1981). Thus, ES cells give a feasible opportunity to produce transgenic animals by cell-mediated gene transfer. Actually, ES cells have been widely used for the alteration of mouse genotypes. Although genetic ma-

본 연구는 과학기술부에서 주관하는 국가선도기술개발과제(HS1690)의 일환으로 수행되었음.

nipulation of ES cells has great economic advantages of improving growth rates, disease resistance, and body and milk composition of domestic animals (Stewart, 1991; Wilmut et al., 1991), the establishment of ES cell lines from these animals has been relatively less studied than that of murine ES cells due to several shortcomings such as long generation time, difficulties in culture and manipulation, etc. Nevertheless, there have been several reports on the isolation of embryonic cell lines from the embryos of farm animals including cattle (Simms & First, 1993; Strelchenko et al., 1991), pig (Evans et al., 1990) and sheep (Notarianni et al., 1991).

Bovine embryonic cell lines can be derived from morula (Strelchenko and Stice, 1994) and blastocyst stage embryos (Simms & First, 1993). In the case of blastocyst embryos, removal of trophectoderm (TE) cells is generally required to efficiently derive putative ES cell lines in domestic animals. Anti-serum (Solter & Knowles, 1975) and calcium ionophore A23187 (CIPA; Onishi & Youngs, 1993) were generally used to selectively remove TE cells from blastocysts. Although chemosurgery using CIPA is convenient to isolate TE-free inner cell masses (ICMs) from blastocysts, the treatment conditions such as concentration and duration have to be carefully controlled because high-dose and/or long-term treatment may cause a toxicity on the viability of the isolated ICM cells. There have been several reports on the isolation of bovine embryonic cell lines using CIPA-chemosurgery. However, the treatment conditions and their results were highly variable depending on the researchers (Onishi & Youngs, 1993; Wada et al., 1994). Therefore, we designed this experiment to determine efficient CIPA-treatment conditions capable of supporting the establishment of pluripotent embryonic cell lines from *in vitro*-developed bovine blastocysts.

MATERIALS AND METHODS

1. Preparation of blastocysts

Ovaries were collected from cows and heifers at a local slaughterhouse and were transported to the laboratory in 0.9% saline at 30 to 35℃. The oocytes were recovered from 2 to 5 mm follicles with 18-gauge needle attached to a 10 ml syringe. The oocytes were cultured in TCM199 supplemented with 10% (V/V) heat-inactivated fetal bovine serum (FBS), 1 μg/ml estradiol and 1 μg/ml FSH-P™ in a four-well multidish at 39℃, 5% CO_2 in air for 22 to 24 h. Then oocytes were fertilized with sperm separated by Percoll Gradient centrifugation in 50 μl droplet of modified Tyrode-Lactate medium (Barvister & Yanagimachi, 1977) containing 2 μg/ml heparin, 20 μM penicillamin, 10 μM hypotaurine, and 1 μM epinephrine. After 18 h of insemination, the embryos were vortexed to partially remove the cumulus cells and cultured in 50 μl droplet of CR1aa medium (Rosenkrans & First, 1991; Rosenkrans et al., 1993) supplemented with 3 mg/ml bovine serum albumin for 2 days. Cleaved (2- to 8-cell) embryos were transferred onto mitomycin C-inactivated mouse embryonic fibroblasts (MEF) in the CR1aa medium supplemented with 1 mM glutamine, 1× Eagle's essential amino acids solution (GibcoBRL), and 10% FBS.

2. Treatment of CIPA

Expanded or hatched blastocysts developed in vitro were washed once in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline. The blastocysts were treated with 50 or 100 μM of CIPA for 25 or 50 min at 39℃. Degraded TE cells were removed by pipetting with finely-pulled Pasteur pipette under dissecting microscope. In case of blastocysts with zona pellucida, the zona was mechanically removed by using of 25-gauge syringe needle. The blastocysts were vigorously blown out to the blade of syringe needle located in the edge of pipette in order to make a slit in the zona pellucida. ICM without TE cells could be extruded out through the slit by vigorous pipetting. The isolated ICMs were transferred onto mitotically-inactivated MEF feeder layer in 4-well multidish to facilitate attachment and subsequent proliferation.

3. Culture conditions of ICMs

After further 2~4 days of culture the proliferated embryonic cells were dislodged and physically torn to small masses using two syringes with 25-gauge needle for subculture. Subsequent subculture was performed at every 5~10 days. The culture medium was Dulbecco's modified

Eagle's medium (DMEM, GibcoBRL) containing 20% ES-qualified FBS (GibcoBRL), 0.1 mM of β-mercaptoethanol, 1× non-essential amino acids (GibcoBRL), 10^3 units of leukemia inhibitory factor (GibcoBRL), 20 μg/ml of insulin (Sigma) and 1× antibiotics (GibcoBRL).

4. Characterization of bovine embryonic cells

The following analyses were preformed using bovine ES-like cells cultured over 10th passages to evaluate their pluripotency.

1) Alkaline phosphatase (AP) staining

AP activity of ES-like cells was determined by using AP diagnostic kit (Sigma) containing TR-red reagent according to manufacturer's instruction.

2) Karyotype analysis

After treated with 0.1 μg/ml of colcemid for 24 h, ES-like cells were trypsinized to single cell suspension. Recovered cells were treated with a hypotonic solution of 0.56% KCl for 20 min, fixed with ice-cold methanol/glacial acetic acid (3:1), and then spread onto clean glass-slide.

3) Induction of differentiation

To test differentiation potency of bovine ES-like cells by suspension culture, the trypsinized cells were allowed to attach on the culture dish for 1 h. Then the bovine ES-like cells could be recovered from the medium because MEF anchored more quickly on culture dish than bovine embryonic cells did. The isolated ES-like cells were suspended into bacteriological petri dish to induce differentiation. After 8 days cultivation appearance of embryoid bodies was examined microscopically.

RESULTS

The colonization rates of blastocysts treated with 100 μM CIPA (15.4 and 4.4% in group I and IV, respectively) were markedly lower than that of 50 μM CIPA-treated groups (51.2 and 47.6% in group I and Ⅲ, respectively) regardless of treatment time (Table 1). In 100 μM CIPA-treated groups, moreover, the detrimental effect of CIPA was remarkable in the long time-treated blastocysts (group Ⅳ), in which only 4.4% of them could colonize. These results suggest that the toxicity of CIPA affected directly on the viability of blastocysts in a dosage- and time-dependant manner. However, it was not clear whether the detrimental effect of CIPA was caused by delivering to ICM via leaky tight junctions among TE cells (Prelle et al., 1996) or after TE degradation. In -treated group I and Ⅲ, a considerable proportions of ICMs colonized, but there was no significant difference between 25 min- and 50 min-treated groups (51% vs. 48%, respectively). However, the overall establishment rate of the embryonic cell lines undergone more than 10 passages was two times higher in the 25 min-treated group than in the 50 min group (4.8% vs. 2.4%, respectively).

Table 1. Effects of CIPA concentration and incubation time for the isolation of bovine ES-like cells from blastocysts

Trt. groups	CIPA Conc. (μM)	treatment Time (min)	No. of blastocysts	No. of ICMs colonized [1](%)	No. of ICMs survived through the subsequent passages[2] (%)										Establishment rate [3](%)
					1	2	3	4	5	6	7	8	9	10	
I	50	25	84	43 (51)	39 (90)	31 (72)	18 (42)	10 (23)	8 (19)	6 (14)	6 (14)	6 (14)	5 (12)	4 (9)	4.8
II	100	25	91	14 (15)	7 (50)	3 (21)	0 (0)								–
Ⅲ	50	50	82	39 (48)	35 (90)	15 (38)	9 (23)	7 (18)	5 (13)	5 (13)	5 (13)	4 (10)	3 (8)	2 (5)	2.4
Ⅳ	100	50	68	3 (4)	1 (33)	0 (0)									–

[1] Colonization rate = no. of ICMs colonized / no. of blastocysts used
[2] Survival rate = no. of ES-colony pools survived / no. of ICMs colonized
[3] Establishment rate = no. of ES-colony pools survived up to 10 passages / no. of blastocysts used

Therefore, these results showed that blastocysts treated with 50 μM CIPA for 25 min could most efficiently develop to embryonic cell lines in this experiment.

We also investigated the survival rate of embryonic cells in each passage throughout successive 10 passages. Although the survival rates of cultures were continuously reduced throughout the passages, a prominent reduction occurred during early passages. In group Ⅲ, especially, a remarkable reduction occurred during second passage and thus the subsequent survival rates from 2nd to 10th passage were not different from those in the group I (2/15 vs. 4/31, respectively). It demonstrates that the toxicity of CIPA treatment is evidently revealed in the starting part of establishment periods. Next, we compared the viability of CIPA-treated blastocysts with that of untreated TE-intact blastocysts to evaluate the effectiveness of CIPA treatment for establishing embryonic cell lines (Table 2).

In blastocysts treated with 50 μM CIPA for 25 min, colonization rate was five times higher than that of intact blastocysts (50% vs. 10%). In addition, CIPA treatment did not show a detrimental effect on the subsequent viability of the colonized embryonic cells because the same proportions of colonized ICMs developed to embryonic cell lines between two groups (5/20 and 1/4, respectively). It means that CIPA treatment conditions defined in this study were highly effective for establishing ES-like cell lines without apparent toxicity of CIPA. Thus, it is essential to remove TE cells from blastocysts for an efficient colonization.

Finally, we examined alkaline phosphatase (AP) activity, capability of embryoid body formation, and euploidism using three ES-like cell lines established independently from CIPA-treated blastocysts. Our ES-like cell lines showed a heterogeneous AP activity (data not shown). The heterogeneous AP activity may be caused by technical difficulty in subculture of embryonic cells at every passage. During splitting ES-like cell colonies, it was difficult to separate the cell mass to single cells by trypsinization because they are tightly associated with each other. Because, in addition, single cells passaged with the use of trypsin could not proliferate or became frequently committed to differentiation, bovine ES-like cells were passaged by a mechanical splitting to small cell masses without a trypsinization. For these reasons, our cell lines may be a mixed cell population with different AP activity. Therefore, we could not conclude whether this heterogeneous AP activity of our ES-like cells came from a intrinsic character of bovine ES-like cells or from heterogeniety in cell populations. We also checked the capability of the ES-like cells for the formation of embryoid body because it is well known as one of the representative characteristics of pluripotent embryonic cells. Expectedly, all the three ES-like cell lines were capable of forming simple embryoid bodies during suspension culture on bacteriological petri dish (Fig. 1). We then examined the chromosomal euploidity of the cell lines by karyotyping (Table 3). The percentages of embryonic cells showing a normal euploidity were 74.5, 79.2 and 68.1% in each cell line and they all showed male genotype (58, XY). Consequently, these results demonstrated that these cell lines have an *in vitro* pluripotency and that our chemosurgery treatment can be safely used to isolate ES-like cell lines from bovine blastocysts.

DISCUSSION

In this report, we demonstrated the usefulness of CIPA-mediated chemosurgery to remove TE cells from *in vitro*-produced bovine blastocysts for the establishment of ES-like cell lines. The blstocysts treated with 50 μM CIPA for 25 min were efficiently developed to embryonic cell lines without loss of the pluripotency.

Removal of TE cells is an essential step for establishment of ES-like cell lines from bovine blastocysts without further

Table 2. Effect of CIPA treatment in the establishment of bovine ES-like cell lines

Treatments	No. of blastocysts used	No. of ICMs colonized (%)	No. of embryonic cell lines established *(%)
Intact blastcysts	38	4 (10.5)	1 (2.6)
CIPA 50 μM 20 min	40	20 (50.0)	5 (12.5)

* Establishment rate = no. of ES-like cell lines established / no. of blastocysts used

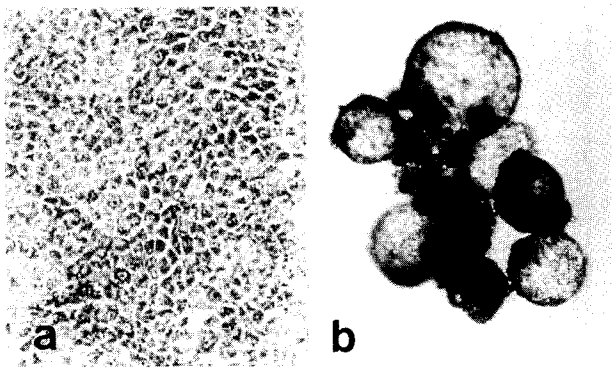

Fig. 1. Differentiation ability of bovine ES-like cell lines *in vitro*. a, bovine ES-like cells grown on MEF feeder layer; b, embryonic bodies differentiated during suspension culture for 8 days

differentiation. For this reason, immunosurgery or chemosurgery was frequently used in TE-removal step. It was reported that while CIPA chemosurgery could remove TE cells from bovine blastocysts at a higher rate than immunosurgery could, the subsequent proliferation was more stable in the immunosurgically isolated ICMs (Prelle et al., 1996). Since we had preliminarily experienced that immunosurgery resulted in an inefficient proliferation of isolated ICMs and its procedure was more complex than that of chemosurgery, CIPA was preferentially used to develop bovine presumptive ES cell lines after optimization of the treatment conditions.

One of the distinguished property of bovine ES-like cells was that individualized cells were not able to colonize and apt to differentiate (Saito et al., 1992). Trypsinization has been successfully used for the subculture of murine ES cells (Evans & Kaufman, 1981). Some researchers have reported successive subculture of bovine (Van Stekelenburg-Haers et al., 1995) and rabbit pluripotent ES cells (Graves & Moreadith, 1993) by trypsinization. However, many attempts using trypsin to passage ES-like cells have led to cell differentiation in cattle (Saito et al., 1992) and rabbits (Giles et al., 1993). We had attempted to apply trypsinization to the passage of primary bovine ES-like cells, but it induced differentiation or no colonization of the cells. Therefore, bovine embryonic cell masses were passaged by tearing into small clumps. From this technical difficulty in subculture, we could not define whether the heterogeneous AP activity of our ES-like cell lines is an intrinsic property of bovine ES cells or not. The AP activity has been found in most pluripotent or presumptive ES cell lines of mice (Robertson, 1987), sheep (Tsuchiya et al., 1994) and pigs (Talbot et al., 1993) except in undifferentiated bovine ES-like cell lines (Polejaeva et al., 1995). Although entire AP activity of ES-like cell lines (Hamano et al., 1996) was reported, it was a result from ES-like cells after 5th passages. AP activity of our ES-like cells undergone 10 passages showed the heterogeneity in AP activity. There was a same result showing heterogeneous AP activity in presumptive bovine ES-like cell lines (Van Stekelenberg-Haers et al., 1995). Therefore, AP activity can not be a critical marker for discerning putative ES-like cells in cattle.

The normal chromosome complement and *in vitro* pluripotency will provide the bovine ES-like cells with a potential usefulness for developmental gene function study and embryology. Moreover, as these cell lines are able to provide an elite route for introducing a foreign gene into the genome of mammals, the potential of pluripotent bovine embryonic cells may be considerable. To introduce a foreign gene into the genome of putative ES cells, DNA-introduction method must be improved. Although germ line chimaeric fetuses were produced from DNA-injected bovine ES cells (Cibelli et al., 1997), the direct injection is a laborious and inefficient method. It was reported that viable bovine and sheep transgenic offspring could be derived from stably-transfected fetal fibroblasts by nuclear transfer (Cibelli et al., 1998; Schnieke et al., 1997). Therefore, provided a scheme for introducing a gene of interest into the

Table 3. Karyotype analysis of ES-like cell lines

Cell lines	Karyotypes	No. of passages	Chromosome count (%)		
			59	60	61
KB-1	Male	10	7 (14.9)	35 (74.5)	5 (10.6)
KB-2	Male	10	8 (15.1)	42 (79.2)	3 (5.7)
KB-3	Male	10	9 (19.1)	32 (68.1)	6 (12.8)

bovine embryo-derived cells, ES-like cells will be another useful cell vector for the production of transgenic animals. Presently, we have the schedule to carry out nuclear transplantation and blastocyst injection in order to determine full potency of the ES-like cell lines and to investigate the possible approaches for gene introduction into genome of bovine embryonic cells.

REFERENCES

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon A, Robl JM (1998) Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 280:1256-1258.

Cibelli JB, Stice SL, Kane JJ, Golueke PG, Jerry J, Dickinson ES, Gao XY, Ponce de Leon A, Robl JM (1997) Production of germline chimeric bovine fetuses from transgenic embryonic stem cells. Theriogenology 47:241 (Abstract).

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154-156.

Evans MJ, Notarianni E, Laurie S, Moor RM (1990) Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. Theriogenology 33:125-128.

Giles JR, Yang X, Mark W, Foote RH (1993) Pluripotency of cultured rabbit inner cell mass cells detected by isozyme analysis and eye pigmentation of fetuses following injection into blastocysts or murulae. Mol Reprod Dev 36:130-138.

Graves KH, Moreadith RW (1993) Derivation and characterization of putative pluripotential embryonic stem cells from preimplantation rabbit embryos. Mol Reprod Dev 36:424-433.

Hamano S, Watanabe Y, Azuma S, Toyota Y (1996) Establishment of embryonic stem (ES) cell-like cell lines derived from bovine blastocysts obtained by *in vitro* culture of oocytes matured and fertilized *in vitro*. In: 13th International Congress on Animal Reproduction, Sydney, Australia, 30 June-4 July, 1996, P21-3.

Notarianni E, Galli C, Laurie S, Moor RM, Evans MJ (1991) Derivation of pluripotent, embryonic cell lines from the pig and sheep. J Reprod Fert (suppl 43) :255-260.

Onishi A, Youngs CR (1993) Comparison of immunosurgery with calcium ionophore treatment for isolation of inner cell masses of porcine blastocysts. Theriogenology 39:274 (Abstract).

Polejaeva IA, White K, Ellis LC, Reed WA (1995) Isolation and long-term culture of mink and bovine embryonic stem-like cells. Theriogenology 43:300 (Abstract).

Prelle K, Stojkovic M, Brem G, Wolf E (1996) Isolation of bovine inner cell masses for the establishment of pluripotent cell lines. 13th International Congress on Animal Reproduction, Sydney, Australia, 30 June-4 July, 1996, P21-6.

Robertson EJ (1987) Embryo-derived stem cell lines. In:Robertson EJ (ed.) Teratocarcinomas and Embryonic Stem Cells: A Practical Approach.Oxford: IRL Press, pp 71-112.

Rosenkrans CF Jr, First NL (1991) Culture of bovine zygotes to the blastocyst stage: effects of amino acids and vitamins. Theriogenology 35:266 (Abstract).

Rosenkrans CF Jr, Zeng GQ, McNamara GT, Schoff PK, First NL (1993) Development of bovine embryos *in vitro* as affected by energy substrates. Biol Reprod 49:459-462.

Saito S, Strelchenko N, Niemann H (1992) Bovine embryonic stem cell-like cell lines cultured over several passages. Roux's Arch Dev Biol 201:134-141.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scotte AR, Ritchie M., Wilmut I, Colman A, Campbell KH (1997) Human factor IX transgenic sheep produced by transfer of neclei from transfected fetal fibroblasts. Science 278:2130-2133.

Simms MM, First NL (1993) Production of fetuses from totipotent cultured bovine inner cell mass cells. Theriogenology 39:313 (Abstract).

Solter D, Knowles BB (1975) Immunosurgery of mouse blastocysts. Proc Natl Acad Sci 72:5099-5102.

Stewart CL (1991) Prospects for the establishment of embryonic stem cells and genetic manipulation of domestic animals. In:Pederson RA, McLaren A, First NL

(eds.) Animal Application of Research in Mammalian Development. New York: Cold Spring Harbor Press. pp 267-284.

Strelchenko N, Stice S (1994) Bovine embryonic pluripotent cell lines derived from morula stage embryos. Theriogenology 41:304 (Abstract).

Strelchenko N, Mitalipova M, Stice S (1995) Further characterization of bovine pluripotent stem cells. Theriogenology 43:327 (Abstract).

Strelchenko N, Saito S, Niemann H (1991) Towards the establishment of bovine embryonic stem cells. Theriogenology 35:274 (Abstract).

Talbot NC, Rexroad CE, Pursel VG, Powell AM (1993) Alkaline phosphatase staining of pig and sheep epiblast cells in culture. Mol Reprod Dev 36:139-147.

Van Stekelenburg-Haers AEP, Van Achterberg TAE, Rebel HG, Flechon JE, Campbell KHS, Weima SM, Mummery CH (1995) Isolation and characterization of permanent cell lines from inner cell mass cells of bovine blastocyst. Mol Reprod Dev 40:444-454.

Wada T, Konishi M, Itakura H, Takedomi T, Aoyagi Y (1994) Study on isolation of inner cell mass from bovine embryos derived from *in vitro* fertilization by exposure to calcium ionophore A23187. J Reprod Dev 40:j53-j57.

Wilmut I, Hooper ML, Simons JP (1991) Genetic manipulation of mammals and its application in reproductive biology. J Reprod Fert 92:245-279.